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Passive transfer of serum from tilapia vaccinated with a *Vibrio vulnificus* vaccine provides protection from specific pathogen challenge

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ABSTRACT

Vibrio vulnificus is a Gram-negative bacterium that has been associated with disease losses in some aquaculture reared fish species. Vaccination has proven effective for reducing the impact of this disease and research has suggested that specific antibodies are important for protective immunity. The present study determined the role of antibodies specific for *V. vulnificus* in protection by passive immunization and identified components of the bacterium the antibodies specifically recognize. Antiserum was generated by vaccinating hybrid tilapia with a formalin killed *V. vulnificus* ARS-1-Br-09 bacterin. Two passive immunization experiments were conducted, with and without heat inactivation of the antiserum. In both experiments, hybrid tilapia (mean weight, 6.5 g) were passively immunized by intraperitoneal injection of antiserum or control serum and then challenged with homologous *V. vulnificus* 24 h post-immunization. Following the challenge, relative percent survival values of 86 and 90 were obtained for tilapia passively immunized with non-heated and heat inactivated antiserum, respectively. Cell lysates and lipopolysaccharide preparations from *V. vulnificus* ARS-1-Br-09 and a heterologous isolate (CECT 4601) were probed with the antiserum and control serum by western blot analyses to determine the specificity of the antibodies. The antibodies exhibited specificity to proteins of both isolates and to the lipopolysaccharide of only the homologous isolate. The results supported a role of specific antibodies in the protection of tilapia against *V. vulnificus*, and suggested that shared immunogenic antigens were involved in protection previously described against heterologous isolates.

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1. Introduction

Vibrio vulnificus is a Gram-negative opportunistic human pathogen that can also cause disease and mortality in some important aquaculture fish species (Austin, 2010). This pathogen has had the largest impact on the production of eels (*Anguilla anguilla* and *Anguilla japonica*) in Europe and Japan (Fouz et al., 2006; Tison et al., 1982); however, there are cases of *V. vulnificus* impacting the production of tilapia *Oreochromis* spp. (Chen et al., 2006; Sakata and Hattori, 1988; Shoemaker et al., 2011) and pompano *Trachinotus ovatus* (Li et al., 2006). The feasibility of vaccination for the prevention of losses in eels due to *V. vulnificus* was established in the laboratory (Collado et al., 2000) and subsequent work demonstrated the efficacy of vaccination under production conditions (Fouz et al., 2001). Recent laboratory studies also demonstrated the effectiveness of vaccination against *V. vulnificus* in tilapia (Shoemaker et al., 2011).

Vaccination of fish against bacterial pathogens typically results in the induction of a specific antibody response and these antibodies are generally presumed to be important for protective immunity, especially against extracellular pathogens. Shoemaker et al. (2011; 2012) demonstrated protective immunity against *V. vulnificus* in hybrid tilapia vaccinated with a formalin killed bacterin. The authors suggested that specific antibodies were involved in the protection due to the observation that vaccinated fish exhibited significantly elevated antibody titers as detected by agglutination. Similarly, other research has suggested that specific antibodies elicited by vaccination against *V. vulnificus* are important for and/or correlated with protection (Collado et al., 2000; Esteve-Gassent and Amaro, 2004; Esteve-Gassent et al., 2003; Fouz et al., 2001). While it is likely that the specific antibodies are important for protection since this has been demonstrated for other *Vibrio* spp. such as *V. anguillarum* (Akhlaghi, 1999), this has not been directly determined for *V. vulnificus* through passive immunization experiments. The objectives of this study were to determine the role of vaccine induced antibodies specific for *V. vulnificus* in protection from experimental challenge in hybrid tilapia and to identify the components of the bacterium the antibodies specifically recognize.

2. Materials & methods

2.1. Fish and rearing conditions

All fish used in this research were apparently healthy sex reversed F1 hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) obtained

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as fry from AQUASAFRA, Inc. (Bradenton, FL, USA). Fish were maintained in a 350 l trough supplied with 28 ± 2 °C dechlorinated municipal water and fed daily (3% body weight) with Aquamax Grower (PMI Nutrition International, Inc., Brentwood, MO, USA). Prior to use in experiments, brain and head kidney tissue from 15 fish were plated onto sheep blood agar (SBA) and incubated at 28 °C for 72 h. All fish were culture negative for *V. vulnificus*. Tilapia used for production of antiserum were maintained in 208 l tanks supplied with the same water source at a flow rate of 0.5 l min^{-1}. Tilapia used for the passive immunization trials were acclimated for 7 d in 57 l tanks filled with static water containing 1.5 g l^{-1} sea salt and were maintained under these conditions for the duration of the bacterial challenge. Half of the water volume was removed daily and then tanks were refilled and sea salt was added to maintain the same concentration. Previous research demonstrated that these conditions were necessary for successful *V. vulnificus* challenges (Shoemaker et al., 2011). All procedures utilizing fish were approved by the USDA-ARS AAHRU Institutional Animal Care and Use Committee.

2.2. Bacterial strains & growth conditions

Two *V. vulnificus* isolates, ARS-1Br-09 and CECT 4601, were used in this research. The isolate ARS-1-Br-09 was isolated from diseased hybrid tilapia (Shoemaker et al., 2011) and isolate CECT 4601 (Spanish type culture collection) was originally isolated from diseased eel (Biosca et al., 1991). Both isolates were previously confirmed as *V. vulnificus* using fatty acid methyl ester analysis and API 20E test (bioMérieux, Inc., Durham, NC, USA) (Shoemaker et al., 2011). ARS-1-Br-09 was used for the production of the vaccine and antiserum and for bacterial challenges. Both isolates were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses. Isolates were resuscitated from −80 °C glycerol stocks and grown for 18 h at 28 °C in tryptic soy broth supplemented with 0.5% sodium chloride (TSB + NaCl).

2.3. Production of antiserum

A formalin killed *V. vulnificus* (ARS-1Br-09) vaccine was prepared as described by Shoemaker et al. (2011) and used for antiserum production in tilapia (mean weight, 9.5 g). One group of 10 tilapia were administered an intraperitoneal (ip) injection with 100 μl of the killed vaccine (1.5×10^8 cfu $fish^{-1}$) and another group of 10 control tilapia were injected ip with the same volume of TSB + NaCl. Nine months following the primary immunization, fish in both groups were booster immunized as described above, and then administered a second booster immunization two weeks afterwards. Eight weeks following the final booster immunization, blood was collected from the caudal vasculature of individual fish from each group using a non-heparinized syringe with a 25 gauge needle. Serum was collected from clotted blood following centrifugation at 3000 ×*g* for 10 min. The agglutinating antibody titer of each serum sample obtained from vaccinated and control fish was determined as described by Shoemaker et al. (2011). Following determination of antibody titers, the serum samples within the vaccinated and control groups were pooled and then the antibody titer of the pooled serum samples was determined. A portion of the serum obtained from vaccinated and control fish was heat inactivated at 56 °C for 1 h.

Table 1
Mean cumulative percent mortality (CPM) ± standard error of the mean (SEM) and relative percent survival (RPS) of hybrid tilapia passively immunized in Trial 1 with antiserum against *Vibrio vulnificus* and control serum and then challenged with *V. vulnificus* ARS-1-Br-09. Average CPM values with different superscript letters indicate a significant difference ($P < 0.05$).

Treatment	Number dead/total	CPM	Average CPM ± SEM	RPS
Control serum	10/10	100	73.3 ± 13.3[a]	–
	6/10	60		
	6/10	60		
Antiserum	2/10	20	10.0 ± 5.8[b]	86
	1/10	10		
	0/10	0		
Mock immunized (PBS)	6/10	60	60	–
Mock challenged	0/10	0	0	–

Table 2
Mean cumulative percent mortality (CPM) ± standard error of the mean (SEM) and relative percent survival (RPS) of hybrid tilapia passively immunized in Trial 2 with heat inactivated antiserum against *Vibrio vulnificus* and heat inactivated control serum and then challenged with *V. vulnificus* ARS-1-Br-09. Average CPM values with different superscript letters indicate a significant difference ($P < 0.05$).

Treatment	Number dead/total	CPM	Average CPM ± SEM	RPS
Control serum	6/10	60	66.7 ± 6.7[a]	–
	7/10	70		
	8/10	80		
Antiserum	0/10	0	6.7 ± 6.7[b]	90
	0/10	0		
	2/10	20		
Mock immunized (PBS)	8/10	80	80	–
Mock challenged	0/10	0	0	–

2.4. Passive immunization and challenge

Two passive immunization experiments (Trials 1 and 2) were performed. In Trial 1, triplicate groups of 10 tilapia (mean weight, 6.5 g) were injected ip with 200 μl antiserum and another triplicate groups of 10 tilapia were injected ip with 200 μl control serum following anaesthetization by immersion into water containing 80 ppm tricaine methane sulfonate (MS-222; Argent Chemicals, Redmond, WA, USA). Additionally, two groups of 10 tilapia each were injected ip with 200 μl phosphate buffered saline (PBS) for use as mock immunized controls in the challenge. The size of tilapia and experimental design for Trial 2 were identical to Trial 1 with the exception that heat inactivated control serum and antiserum were used.

At 24 h post-passive immunization, tilapia were challenged with *V. vulnificus* ARS-1-Br-09. Prior to the challenge in Trial 1, fish were selected from the antiserum and control serum tanks (n = 5 fish per group), anaesthetized by immersion into water containing 80 ppm MS-222, and blood was non-lethally collected and fish were returned to the tanks. Serum was collected for determining agglutinating antibody titers as previously described. Then, fish were anaesthetized and challenged by ip injection with 100 μl volume containing 2.7×10^7 cfu $fish^{-1}$. One group of 10 mock immunized tilapia were challenged with *V. vulnificus*; while the other group was mock challenged by ip injection with 100 μl of TSB + NaCl and served as the negative control. In Trial 2, the *V. vulnificus* challenge was conducted identically with the exception that blood was not collected from fish prior to challenge and fish were challenged with 3.0×10^7 cfu $fish^{-1}$. In both trials, mortalities were recorded daily for 15 d and brain tissue from a minimum of 30% of the dead fish was plated onto SBA plates for re-isolation of *V. vulnificus*. Plates were incubated at 28 °C for 72 h and presumptive *V. vulnificus* colonies were identified by fatty acid methyl ester analysis (Shoemaker et al., 2005) and API 20E test. The mean cumulative percent mortality (CPM) was calculated for each group and the relative percent survival (RPS) was determined as described by Amend (1981).

2.5. Preparation of cell lysates and lipopolysaccharides

Cell lysates of *V. vulnificus* ARS-1Br-09 and CECT 4601 were prepared by resuspending 125 mg (wet weight) of cells into 900 μl sterile water. The bacterial solutions were then transferred to Lysing Matrix B tubes (MP Biomedicals) and homogenized for 5 min in a FastPrep®-24 (MP Biomedicals) instrument with cooling on ice for 1 min after each minute

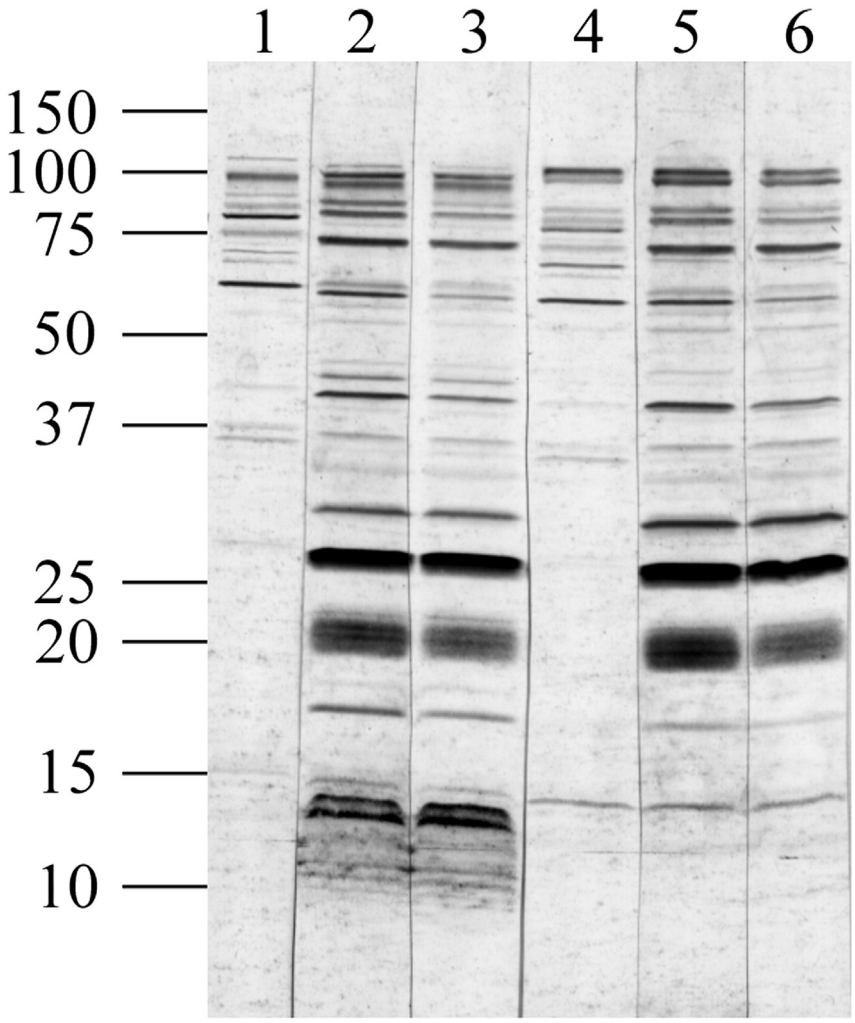

Fig. 1. Western blot analysis of cell lysates of *Vibrio vulnificus* ARS-1-Br-09 (Lanes 1–3) and *V. vulnificus* CECT 4601 (Lanes 4–6). Antigens were probed with control serum (Lanes 1 and 4), antiserum (Lanes 2 and 5), or heat inactivated antiserum (Lanes 3 and 6). Molecular mass markers (kDa) are indicated on the left.

of homogenizing. Tubes were centrifuged at 16,000 ×*g* for 30 min at 4 °C, and the supernatant was collected and stored at −20 °C. Protein concentrations were determined using a Micro BCA™ protein assay (Pierce). Lipopolysaccharides (LPS) from each isolate were prepared following the method of Hitchcock and Brown (1983) as modified by LaFrentz et al. (2004).

2.6. SDS-PAGE & western blot analyses

Cell lysates and LPS preparations from *V. vulnificus* ARS-1-Br-09 and CECT 4601 were probed with the antiserum and control serum used for passive immunization by western blot analyses to determine the specificity of the antibodies. Proteins within cell lysates and LPS preparations were separated by SDS-PAGE as described by LaFrentz et al. (2004). Proteins (10 μg) or LPS preparations (20 μl) were separated in precast 12.5% polyacrylamide gels using a Criterion™ Cell (Bio-Rad). Proteins and LPS resolved by SDS-PAGE were transferred to nitrocellulose membranes by electrophoresis at 100 V for 1 h in a Criterion™ blotter (Bio-Rad) according to the manufacturer's directions. Western blot analyses were performed as described by Shoemaker et al. (2010).

2.7. Statistical analysis

The parametric unpaired t-test procedure of GraphPad Prism version 6.05 (GraphPad Software, La Jolla, CA, USA) was used to compare the mean cumulative percent mortality between tilapia passively immunized with antiserum and control serum (Trial 1) or heat inactivated antiserum and heat inactivated control serum (Trial 2). Differences were considered significant at $P < 0.05$.

3. Results & discussion

Antiserum was generated by vaccination of hybrid tilapia with a formalin killed *V. vulnificus* bacterin and used for passive immunization to test the hypothesis that vaccine induced specific antibodies have a role in protective immunity against *V. vulnificus* challenge. The pooled antiserum exhibited an agglutination antibody titer of 128, the control serum exhibited a titer of <4, and these were used for passive immunization. Twenty four hours post-immunization and just prior to *V. vulnificus* challenge in Trial 1, serum was collected from passively immunized fish to determine antibody titers. The mean agglutination antibody titers from fish passively immunized with the antiserum and control serum were 83.2 and <4, respectively. The presence of these specific antibodies in tilapia passively immunized with the antiserum was correlated with protection from *V. vulnificus* ARS-1Br-09 challenge (Table 1). The mean CPM observed for these fish (10 ± 5.8%) was significantly ($P < 0.05$) lower than the CPM (73.3 ± 13.3%) of fish passively immunized with control serum, and a relative percent survival of 86 was obtained. No mortalities occurred in the mock challenged control group, and the CPM of the fish mock immunized with PBS was 60%, similar to the CPM of fish injected with control serum (Table 1).

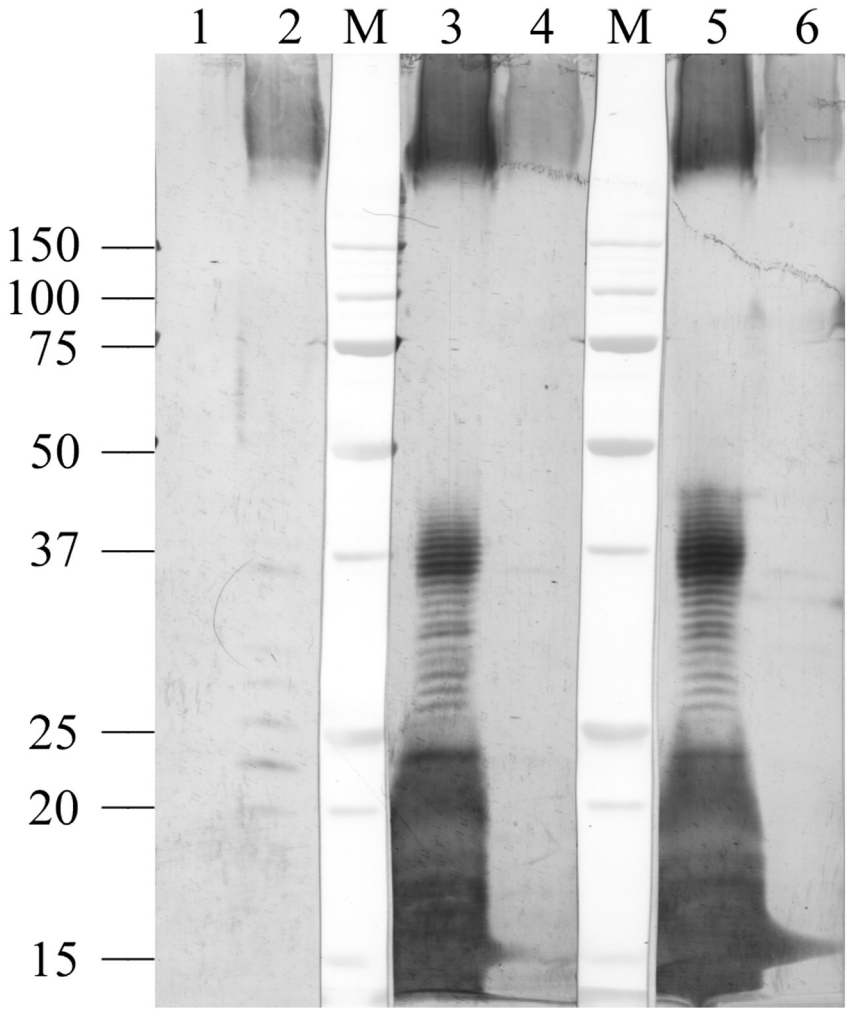

Fig. 2. Western blot analysis of lipopolysaccharide preparations of *Vibrio vulnificus* ARS-1-Br-09 (Lanes 1, 3, and 5) and *V. vulnificus* CECT 4601 (Lanes 2, 4, and 6). Antigens were probed with control serum (Lanes 1 and 2), antiserum (Lanes 3 and 4), or heat inactivated antiserum (Lanes 5 and 6). Molecular mass markers (kDa) are indicated on the left and shown in Lanes M.

V. vulnificus was re-isolated from the brain tissue of all challenge mortalities examined ($n = 14$).

The results from Trial 1 suggested that antibodies specific for *V. vulnificus* are important for protective immunity; however, heat labile non-specific immune factors present in the serum may have contributed to the observed protection. Therefore, in Trial 2, the antiserum and control serum were heat treated to inactivate non-specific immune factors such as complement. Following *V. vulnificus* challenge, tilapia passively immunized with the heat inactivated antiserum exhibited a CPM of $6.7 \pm 6.7\%$ which was significantly ($P < 0.05$) lower than the CPM of tilapia injected with control serum ($66.7 \pm 6.7\%$; Table 2) and a RPS of 90 was obtained. No mortalities occurred in the mock challenged control group, and the CPM of the fish mock immunized with PBS was 80% (Table 2). *V. vulnificus* was re-isolated from the brain tissue of all challenge mortalities examined ($n = 10$). The results obtained in Trials 1 and 2 were similar which suggested that heat labile immune factors present in the transferred antiserum were unlikely to have been involved in the protection observed. Additionally, in both trials the CPM of the fish mock immunized with PBS and then challenged was similar to the CPM of the tilapia injected with control serum, further supporting this observation. Although the contribution of heat resistant immune factors to the protection observed was not determined, the results support a likely role of the specific antibodies elicited by vaccination in protective immunity. Further, detection and quantification of specific antibody against *V. vulnificus* in vaccinated fish may be useful metrics for predicting vaccine efficacy in the field.

Previously, Shoemaker et al. (2011) demonstrated that the *V. vulnificus* bacterin provided protection against challenge with the homologous vaccine isolate (ARS-1Br-09) as well as a heterologous isolate (CECT 4601). Therefore, cell lysates and LPS preparations from both *V. vulnificus* isolates were probed with the antiserum and control serum in western blot analyses to determine the specificity of the antibodies. The results demonstrated that the antibodies present in the antiserum exhibited specificity with a number of proteins present in the cell lysates of both isolates (Fig. 1). In fact, there were few differences in immunoreactive bands between the isolates with the exception of antigens between 10 and 15 kDa (Fig. 1), which suggested antigenic similarity of these isolates. Although the proteins in the present study were not identified, previous research has shown that outer membrane proteins of different isolates of *V. vulnificus* are antigenically similar (Biosca et al., 1993). In contrast, the antiserum exhibited antibodies specific for the LPS of the homologous isolate (ARS-1Br-09) but no reactivity to the LPS was observed for the heterologous isolate (Fig. 2). This may suggest that these two isolates have different LPS serotypes. At least five different LPS serotypes have been previously described (Amaro et al., 1992; Martin and Siebeling, 1991). The control serum exhibited antibody specific for a few proteins of both isolates (Fig. 1) and minor reactivity to a few antigens in the LPS

preparations (Fig. 2). Heat inactivation of the sera did not affect western blot results (Figs. 1 and 2). Taken together, these results demonstrate that the antibodies present in the antiserum exhibited specificity to both protein and LPS components of *V. vulnificus*, similar to previous research in eels vaccinated against *V. vulnificus* (Esteve-Gassent and Amaro, 2004). The shared immunogenic antigens between the homologous vaccine isolate and heterologous isolate (CECT 4601) may explain the ability of the formalin killed bacterin to provide protection against both isolates as shown by Shoemaker et al. (2011).

4. Conclusions

Previous research has suggested that antibodies specific for *V. vulnificus* are important for immunity due to the association of elevated specific antibody titers and protection. The results of the passive immunization experiments in the present study supported a role of specific antibodies in protection of tilapia against *V. vulnificus*, and suggested that shared immunogenic antigens are involved in the protection against heterologous isolates.

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